Cytokine gene polymorphisms in sudden infant death

By

Linda Ferrante

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Institute of Forensic Medicine

University of Oslo, Norway

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"Live as if you were to die tomorrow.
Learn as if you were to live forever."
Mahatma Gandhi

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List of papers

This thesis is based on the following papers:

I. Ferrante L, Opdal SH, Vege Å, Rognum TO. TNF-α promoter polymorphisms in sudden infant death. Hum Immunol 2008; 69:368-73.

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III. Ferrante L, Opdal SH, Vege Å, Rognum TO. IL-1 gene cluster polymorphisms and sudden infant death syndrome. Hum Immunol 2010; 71:402-6.

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1 Introduction

1.1 History of Sudden infant death syndrome (SIDS)

For centuries sudden infant death syndrome (SIDS) has been a subject of mystery and superstition. It has been known for at least 3000 years, and is mentioned in the Bible. Unexpected child death was thought to be caused by overlaying during the times of King Solomon. The biblical tale describes two women who lived in the same house, both had a son. One night one of the boys died because the mother had laid on him. She quickly changed her dead boy with the live boy of the other woman. As they woke up the women started to quarrel about which of them that was the mother of the live child. They brought their case to King Solomon who acted as if he was to cut the boy in two pieces so that each women could receive one half of the baby. When the women heard this verdict one of them cried out “Please, My Lord, give her the live child - do not kill him!” while the other woman replied "It shall be neither mine nor yours - divide it!". The king then realized that the true mother was trying to save the child and gave her the baby (figure 1) (*1*).

Figure 1: King Solomon passing judgment on who is the rightful mother to the baby. Source: Nineteenth century engraving by Gustave Doré.

To avoid such situations, the Greek physician Soranus of Ephesus wrote in the first medical textbook instruction to the mothers not to share bed with their babies. The theory of overlaying remained the only explanation for SIDS in centuries.

In the late 1800s and early 1900s physicians was convinced that cot death was caused by a pathologically enlarged thymus which would inflict internal suffocation. It was believed that the enlarged thymus could asphyxiate the child from inside (*2*). To prevent suffocation the infants were radiated to reduce the thymus size. This gave an immediate reduction, but within days it retained its normal size. This theory was widely accepted and referred to as status thymolymphaticus, and was not rejected until the 1940s, when it was discovered that a large thymus represents a normal developmental stage (*3*).

In the 1950s the trend shifted and a paper claiming viral and bacterial inflammation in the upper airways as a cause of death was published (*4*). This study investigates 26 infants below 12 months of age whose death was observed by an adult. In ten of the cases the gross autopsy findings were sufficient to explain death; nine cases died due to a heart disease while in one case interstitial bronchopneumonia was found (*4*). In the 16 cases were gross autopsy findings were inconclusive, microscopic investigations revealed that bronchitis was present in 11 of the 16 cases (*4*). Using current diagnostic criteria, most likely some of these 16 cases would be given a diagnosis of SIDS. Another theory was hypogammaglobulinemia (*5*). Spain and coworkers measured postmortem serum gamma globulin in three infants who died suddenly and unexpectedly, and found that these infants had low gammaglobulin levels compared to infant controls (*5*). However, this assumption was made based on very few observations, with only three cases and two controls. A larger study later that decade did not reproduce the findings, but showed similar levels of gamma globulin in babies found dead suddenly and unexpectedly as well as in healthy babies (*6*).

The 1960s was the decade when researchers proposed that the cause of sudden infant death was hypersensitivity to cow's milk (*7*). They claimed that these infants had elevated levels of antibodies to cow's milk in their sera, and showed that guinea pigs with similar levels of antibodies could be killed by an anaphylactic reaction by introducing a small amount of milk in their pharynx. However, Gold et al was unable to confirm the finding (*8*), and the hypothesis was soon abandon.

In 1969 the “syndrome” was introduced by Beckwith (*9*) and the term sudden infant death syndrome - SIDS - was given its ICD code (798). During the 1970s much focus in SIDS research was directed towards the apnea theory (*10*). Several prominent researchers investigated this theory, and in 1972 Steinschneider published a study in which he claimed that the prolonged apnea was part of the final death mechanism in SIDS (*11*). As a result of this research apnea monitors were developed and distributed world wide, as an attempt to prevent SIDS. However, it later appeared that two of the five infants described by Steinschneider as dying from SIDS after episodes with apneas in fact were homicides.

Naeye investigated a wide range of tissue specimens from SIDS victims and age matched controls, and found seven tissue markers for hypoxia and hypoxemia significantly more frequent in SIDS than in controls (*12*). These were hypertrophy and hyperplasia of the smooth muscular media in the walls of the small pulmonary arteries, right ventricular hypertrophy of the heart, prolonged retention of periadrenal brown fat, excessive production of red blood cells in the liver, hyper- or hypoplasia of glomic tissue in the carotid body, hyperplasia of chromaffin tissue in the adrenal medulla, and brain stem scarring. Three of these markers were later verified by other researchers; excess of brown fat around the adrenals, excessive production of red blood cells in the liver and scarring in the brain stem (*13, 14*). The latter observation was confirmed in a multi center study (*15*). Astrogliosis of the brainstem has been confirmed by several research groups whereas some groups have not been able to reproduce the findings (*16*). Interestingly, in a series of SIDS cases from the Institute of Forensic Medicine in Oslo, astrogliosis of the brain stem was associated with maternal smoking during pregnancy (*17, 18*).

The presence of brainstem astrogliosis may also point to episodes of hypoxia after birth but prior to the fatal event. Since this tissue markers takes several days to develop their presence cannot throw light on the period immediately prior to death. Thus, the discovery of increased hypoxanthine levels in vitreous humor of SIDS victims attracted much attention (*19*). Hypoxanthine is a biochemical marker of hypoxia and indicates that a large proportion of SIDS victims have had repeated hypoxic episodes immediately prior to death (*20*). Evidence of antecedent hypoxia is further substantiated by Jones et al in 2003 by demonstrating increased concentrations of vascular endothelial growth factor (VEGE) in the cerebrospinal fluid (CSF) in SIDS victims (*21*).

In the 1980s morphological studies of the central nervous system in SIDS victims were given priority. The hypothesis of immature neuronal respiratory control in SIDS were proposed by Quattrochi et al in 1980 (*22*). Hannah Kinney's research group demonstrated delayed myelinization in brains from SIDS victims (*23*). In the late eighties a large epidemiological study was performed by NICHD were several risk factors were introduced, among them were low birth weight, illness after birth and lack of sufficient medical care (*24*). As a result of this study, NICHD in 1993 published the Histophatological Atlas of the sudden infant death as a tool to standardize the diagnosis (*25*). In the Nordic countries a similar approach was initiated by the Nordic council of ministers (*26, 27*). In the Nordic study (Nord-SIDS) focus were on risk factors as prone sleeping, smoking, warm environment and on standardizing the SIDS diagnostic.

1.1.1 Environmental risk factors – the great turn

The dominating discovery in the early 1990s was the dramatic decline in SIDS deaths reported from countries all over the world when the babies were put to sleep supine instead of prone (*28-31*). This was based on discoveries done in the late 1970s, when dr. Tonkin in New Zealand and dr. Beal in Australia visited families that had lost infants in SIDS and learned that the main common feature was that the babies had slept prone (*32-34*). In January 1990 a Norwegian pediatrician, professor Markestad from the university of Bergen, issued a written advice against a prone sleeping position. In the late 80's 65% of all infants had been put to sleep prone, eight months after Markestad gave the new instruction, prone sleeping was drastically reduced to only 7% of the infants (*35*). The effect on the Norwegian SIDS rates was immense (fig. 2), the rate in 1991 being 50 % lower then the 1989 rate (fig. 2)

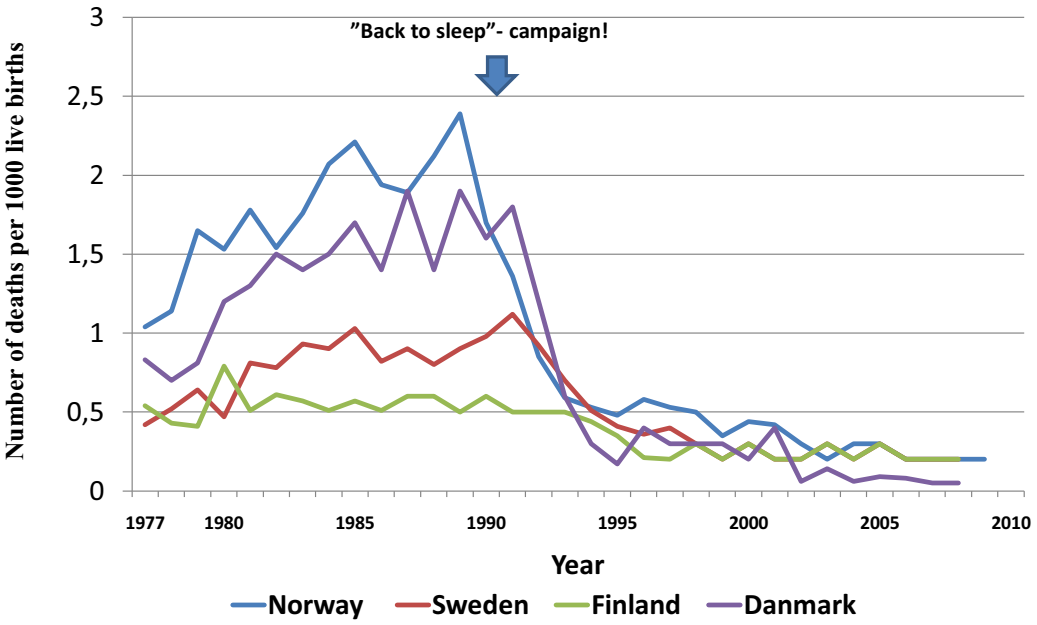


Figure 2: SIDS-rate in the Nordic countries 1977-2008. The campaign to turn the babies from prone to supine sleeping was in Norway initiated by professor Trond Markestad in Bergen at the end of 1989, and was introduced in the other Nordic countries in March 1990. The effect was dramatic (36).

Kohler and Markestad stressed that the information to parents should focus on three well known risk factors; parental smoking, prone sleeping position and ambient temperature (*37*). Markestad also reported the effect on this type of information and showed that within only a few months the parents and caregivers were now putting their babies to sleep on their back (*38*). In 1992, the American Academy of Pediatrics in USA issued a recommendation that infants with no medical complications should be put to sleep on their side or their back (*39*).

1.1.2 Genetic risk factors

Another research area that received a lot of attention during the 1990's was an observation made already in the 80s on the enzyme medium chain acylcoenzyme A dehydrogenase (MCAD). MCAD is an enzyme involved in the β oxidation of fatty acids in mitochondria. A case report from Sheffield describes an 18 month old boy that first was thought to be a SIDS victim, but after screening for this enzyme deficiency it was found that the boy suffered from MCAD- deficiency (*40*). The most common disease causing mutation in the MCAD gene is the A985G, and this mutation have been investigated in SIDS populations all over the world (*41-49*). However, the G985 allele does not seem to be overrepresented in SIDS.

Besides MCAD, the gene encoding complement component C4 was one of the first genes to be investigated with regard to SIDS (*50-52*). The gene consists of two loci, C4A and C4B, that are highly polymorphic, and even a partial deletion of one of the alleles may cause an immune response that is not able to neutralize bacteria adequately. Studies from Norwegian and German SIDS populations did however not uncover any association between SIDS and C4 deletion, but both studies report an association between partial deletions of the C4 gene and a history of slight infection prior to death in SIDS (*50-52*).

During the last decade SIDS research has been increasingly focused on the study of genetic predisposing factors, and so far genes involved in the regulation of the immune system, cardiac function, the serotonergic network and brain function and development have emerged as the most important with regard to SIDS (*53*). Various mutations and polymorphisms in these genes have been found associated with SIDS, but so far only mutations in genes involved in long QT syndrome (LQTS) may be viewed as independent cause of death. Concerning the other possible genetic predispositions to SIDS, they are most likely part of a polygenic inheritance pattern, contributing to triggering a death mechanism when combined with environmental risk factors, such as maternal smoking, prone sleeping position or infection.

Long QT syndrome (LQTS) is a genetically heterogeneous cardiac disorder that is caused by mutations in several genes coding for ion channels, including KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, KCNJ2 and CAV3. The rationale for investigating LQTS genes in SIDS is based on a study by Schwartz et al. They screened over 30 000 newborns with a one year prospective follow-up, and found that

prolonged QT interval in the first week of life was strongly associated with sudden infant death (*54*). A large study of 201 Norwegian SIDS cases demonstrated that 9.5% of cases diagnosed as SIDS carried functionally significant genetic variants in the LQT genes, indicating that sudden arrhythmic death is an important contributor to the group of sudden unexpected deaths in infants (*55*). The most commonly observed mutation was in the SCN5A gene, which is of particular interest since mutation in this gene is known to cause arrhythmias during sleep, when most SIDS deaths occur. Due to the finding of LQTS mutations in SIDS the autopsy protocol in Oslo was extended to include screening of LQTS genes. Consequently, infants with such genetic disorder are not included in the SIDS group.

Table 1: Changes in SIDS research interest over decades

Time	Hypothesis
Bible	Maternal overlaying
1890	Thymolymphaticus
1950	Hypogammaglobulemia
1960	Hypersensitivity to milk
1970	Apnea
1980	Tissue- and biochemical markers for hypoxia
1990	Prone sleeping/environmental risk factors, immunological "overreaction"
2000-	Seretonergic imbalance, Genetic risk factors

1.2 SIDS definition

The first SIDS definition was established during the conference in 1969, initiated by the National institute of Child Health and Human Development (NICHD). The purpose was to form a consensus for a common classification, and resulted in the definition formulated by Beckwith: "The sudden death of any infant or young child, which is unexpected by history, and in which a thorough post mortem examination fails to demonstrate an adequate cause of death" (*9*)

The most widely used definition for SIDS today is the San Diego definition, which was approved at the 8th SIDS International Conference, in Edmonton, Canada,

in 2004. According to this definition, SIDS " is defined as the sudden unexpected death of an infant under 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation that includes carrying out a complete autopsy and review of the circumstances of death and the clinical history" (*56*).

This definition divides SIDS into three categories. These are named category I and II SIDS and in addition there is a category for unclassified SIDS. The definition have detailed requirements for autopsy findings, clinical history and circumstances of death.

Category I SIDS includes infants that meet the general definition and are between the age of 21 days and 9 months, and this category also requires a full term pregnancy and normal growth. Investigation of the scene were the baby was found dead is required, in order to rule out any environmental cause of death. A post mortem examination must be performed, and no pathological findings or any sort of trauma that might explain death must be uncovered. Likewise, there must be negative toxicology.

The category II SIDS includes the infants that meet the category I criteria except one or more of the following; age range outside category I, similar deaths in near family, neonatal or perinatal conditions as for example premature birth, circumstances of death not clarified and findings at autopsy.

The unclassified SIDS is the cases in which the category I and II were not met, or an alternative diagnosis is equivocal. Infants that did not undergo a post mortem examination will also be included in this category. Many forensic pathologist have great doubt about the latter category – giving the case a SIDS diagnosis without performing a post mortem examination. The fact that in genuine SIDS recent studies only comprise approximately 40% of the infants that suffer sudden unexpected deaths, makes it even more important to perform an autopsy (*57*) .

The diagnostic criteria used in SIDS diagnostic vary worldwide, providing a challenge to researchers. This fact makes it difficult to compare SIDS rates between countries and to perform international collaboration studies including different SIDS populations. Due to the high SIDS rate in the Nordic countries during the 80'ties, especially in Norway and Denmark, a Nordic SIDS study was initiated by the Nordic Council of Ministers (*27*). As part of this NORD SIDS study forensic pathologists from all Nordic countries gathered and developed a protocol for the classification of

SIDS. There were three categories, pure SIDS, borderline SIDS and non-SIDS. To meet the diagnostic criteria for pure SIDS the autopsy and clinical information must not reveal any pathology. The borderline SIDS group includes cases with pre-existing congenital disorders, clinical symptoms and/or post mortem findings not sufficient to be the cause of death. For the non-SIDS cases the cause of death is established, either by clinical information and/or post mortem findings. The term unclassified is not used, and thus a few unclassified cases are named borderline SIDS.

Since 1992 all SIDS cases have been evaluated according to the Nordic criteria at the Institute of Forensic Medicine in Oslo. Cases from the period 1984-1992 were re-classified blindly using the new classification criteria, which lead to change of diagnosis in 10% of the cases (*58*). Cases of sudden unexplained deaths of infants from the second week after birth until week 52, were included in the diagnostic. A few of the small children who suffered sudden unexpected and unexplained death were also included in the diagnostic. They comprise 13% of the total SIDS-population and are now classified as sudden death in small children (SUDIC) (*56*).

1.3 SIDS and infection

In 1947 Werne et al suggested that respiratory infection was the cause of death in an otherwise healthy infant (*59*). Since then, there has been numerous reports and papers describing signs of slight infection in SIDS infants. An immunological "overreaction" has been postulated since about half of the SIDS victims have had symptoms of slight infection in the days before death (*60, 61*).

Arnon et al (1978) have hypothesized that some infants might suffer respiratory arrest due to botulinium toxin produced by Clostridium Botulinum (*62*). From a cohort of 280 infants they showed that botulinum toxin was present in 10 infants, of which 9 had been diagnosed as SIDS (*62*).

Stoltenberg et al have reported immune stimulation in both the upper airways and intestines, showing that SIDS had higher number of IgM immunocytes in the tracheal wall than controls, but significantly lower numbers of IgA and IgM immunocytes than cases of infectious death (*63*). In the duodenal mucosa the number of IgA immunocytes was higher in SIDS cases than in controls (*63*). These findings indicate that the mucosal immune system is activated in a large proportion of SIDS. It is also shown that SIDS have higher IgG and IgA-immunocyte density in the palatin tonsillar

compartments than controls (*64*). Furthermore, salivary glands have a higher number of CD45+ stromal leukocytes, as well as intensified epithelial expression of HLA-DR and secretory component, and increased endothelial expression of HLA class I and II (*65*). These observations confirm that the immune system is activated in SIDS, probably with release of certain cytokines that are known to up-regulate epithelial expression of HLA-DR and secretory component (*65*).

A real breakthrough for the immunological overreaction theory was the demonstration by Vege et al (*66*), who showed that SIDS victims who have had signs of slight infection prior to death had both increased number of IgA-immunocytes and HLA-DR expression in their laryngeal mucosa, as well as increased levels of IL-6 in their cerebrospinal fluid (CSF) (Figure 3). In fact, half of the SIDS victims had CSF IL-6 concentrations in the same range as victims of meningitis and septicemia (*60*). A further support for the infection theory is a study performed on registry data from Norway and Sweden which suggests that there is a co-variation between epidemics of whopping cough and SIDS (*67*). The association was stronger in Sweden than in Norway, which may reflect that Swedish infants not are vaccinated against Bordetella pertussis while the Norwegians infants are (*67*).

Recently, Stray-Pedersen et al showed that SIDS victims with positive Helicobacter pylori Stool Antigen (HpSA) immunoassay had elevated IL-6 in the cerebrospinal fluid compared to SIDS victims with negative HpSA test (*68*). Furthermore, detection of helicobacter pylori antigen in stool was found associated with SIDS and death due to infection, indicating that this bacteria may represent a contributing factor to sudden death during the first months of life (*68*).

Surfactant protein A (SP-A) is a protein produced in the lungs, with a major purpose to reduce the surface tension at the alveolar air-liquid interface. Furthermore, it takes part in regulation of the inflammatory process. Interestingly, with regard to SIDS there is a drop in alveolar SP-A expression in the first months after birth (*69*), corresponding to the classical age peak of SIDS. Thus it may be hypothesized that this transient low expression of SP-A may be a part of the increased vulnerability for SIDS at that age (*69*).

It is also suggested that Staphylococcus aureus (S.aureus) are involved in events leading up to SIDS (*70*). Based on observations from samples collected from the intestinal tract in SIDS compared with samples from feces from a group of healthy controls, it was shown that S. aureus and staphylococcal enterotoxins were more

prevalent in SIDS (*70*). However, as much as 40% of the controls were positive for S.Aureus, indicating that this bacteria is common in infants, and that the detection may not be seen as a support for the diagnose of SIDS.

Another study investigated pyrogenic toxins of S.aureus in SIDS infants from different countries (*71*). The study reported these pyrogenic toxins in > 50% of SIDS infants from three different countries; Scotland, France and Australia, and suggest that further investigation into the effect of the toxins may be important (*71*). A study by Blackwell et al found that the prevalence of the aureus in nasopharyngeal flora was significantly higher in SIDS cases compared to age matched healthy controls (*72*). Furthermore, SIDS found in a prone sleeping position more often had symptoms of slight infection prior to death than the babies put to sleep on their back.

The level of IgA imunocytes in larynx are found to be higher in SIDS victims than in control. (*66, 73*). Furthermore, the relationship between laryngeal immune stimulation, clinical signs of slight infection prior to death and high levels of IL-6 in CSF (*66*) may indicate an interaction between the immune system and the central nervous system (CNS). The assumption of such a relationship is strengthened by the recently reported increased IL-6 receptor expression on serotonergic cells in brain stem nuclei involved in respiratory regulation in SIDS cases compared to controls (*74*). The fact that a large proportion of SIDS victims whose brain stems had been investigated had had a common cold prior to death, strengthens the hypothesis that there may be a connection between activation of the mucosal immune system and a dysfunction of the seretonergic network regulating respiration and heart function in SIDS.

In summary, these studies indicate that a significant proportion of SIDS victims have an activated immune system (Figure 3) that seems to cause autonomic dysregulation inducing down-regulation of respiration, irreversible hypoxia and death. Scares in the brain stem of SIDS victims indicate either a predisposition acquired before birth (*75, 76*) or they represent sequela after a previously survived apparent life threatening event (ALTE) (*77*). Increased concentration of hypoxanthine in vitreous humour and urine of SIDS victims (Figure 3) represent repeated episodes of hypoxia shortly before death (*19, 20, 76, 78-80*). A possible pathogenesis of SIDS may be summarized by a vicious circle triggered by environmental factors such as a common cold combined with prone sleeping position. An immune reaction out of homeostatic control induces serotonergic imbalance which consequently leads to

impaired breathing, hypoxia and death (*73, 81*). The purpose of this thesis is to search for genetic factors that may explain the fatal disturbances of this immunological homeostasis.

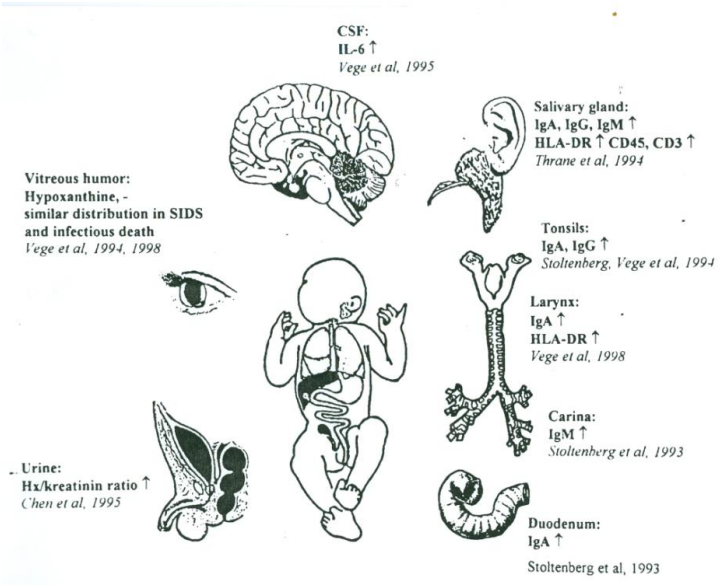


Figure 3: The salivary glands, tonsils, laryngeal and duodenal mucosa show increased number of immune producing cells compared to controls (63-65). The number of IgA-immunocytes and HLA-DR expression are increased in laryngeal mucosa, and the CSF show elevated IL-6 concentration (66). Vitreous humour and urine contain increased amount of hypoxanthine due to ante mortem hypoxia (20, 78, 80)

1.4 Cytokines

The discovery of cytokines was initiated by research trying to understand the pathogenesis of fever. Cytokines are specialized peptides, proteins, and glycoproteins that are produced by different immune cells, for instance when exposed to a pathogen. Important functions of the cytokines are to supervise cell communication and regulate the cells activity during the inflammatory process, making the cytokines important intercellular mediators. The different cytokines and their system of mutual interaction are known as the cytokine network, and until now at least 36 different human cytokines have been detected. The cytokines may be subdivided into different groups of proteins, and most important are the interleukins (IL), the tumor necrosis factors (TNF) and the interferons (IFN), which all are highly involved in the immunological process during an infection. The cytokines can act in a paracrine and autocrine manner, and are commonly divided into pro-inflammatory and anti-inflammatory cytokines.

Interleukins are signaling molecules that were first discovered as products of leukocytes, but they are also produced by other immune cells such as monocytes, macrophages and endothelial cells. Several interleukins have been described, and they all have a specific involvement in the immunological process.

1.5 Pro-inflammatory cytokines

The pro-inflammatory cytokines are immunoregulators that favor inflammation. The most important are IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18, TNFα and IFNγ. These cytokines act as endogenous pyrogenes (IL-1, IL-6, TNFα), up-regulate the synthesis of secondary mediators and other pro-inflammatory cytokines produced by both macrophages and mesenchymal cells (including fibroblast, epithelial and endothelial cells), stimulate the production of acute phase proteins, and attract inflammatory cells.

1.5.1 Interleukin 1α

Interleukin 1α (IL-1α) is an epidermal cytokine produced continuously by monocytes, tissue macrophages, keratinocytes and other epithelial cells. IL-1α is found in large

concentrations in the epidermis, which is due to the essential role of IL-1α preventing entry of microorganisms through the skin, and helping the lymphocytes to fight infection. IL-1α is also responsible for the migration of leukocytes through the blood vessels, and causes fever by affecting the thermo-regulating area of the brain. The protein is quite large, and it is stored in the cells until it is cleaved into a mature version of the protein. The gene encoding IL-1α is located on the long arm of chromosome 2 (cytogenetic location 2q14). The most commonly investigated genetic variations in the IL-1α gene are the SNPs in base pair +4845 and +889 relative to upstream transcription start. The IL-1α +889 polymorphism have been shown to alter the production of IL-1α (*82*), while the polymorphism in +4845 is thought to affect the sensibility to calpains, and the ability to cleave the pro-protein into the active form (*83*). It has been shown that the +4845T allele occur more frequent in patients with chronic polyarthritis, and also that +4845 is associated with polymyalgia rheumatica (*84, 85*). A VNTR in intron 6 of the gene has also been reported, and even though it has not been reported any simple clear-cut correlation between IL-1α production and number of repeats it has been suggested that IL-1α production decreases with increasing number of the intronic repeat sequence (*86*).

1.5.2 Interleukin 1β

Interleukin 1β (IL-1β) is produced as a pro-protein by monocytes, activated macrophages and dendritic cells, and it is an important mediator in a variety of cellular responses in the immune-activated cell. The most important roles of the IL-1β are cell proliferation, differentiation, and apoptosis. Even just a small amount of IL-1β will induce fever and hypotension, and stimulate the production of other cytokines. Two polymorphisms of particular interest within the gene have been reported, the SNPs -511C/T and +2018T/C. Regarding -511C/T it is shown that the T allele will induce increased production of IL-1β, while the C allele might influence the production in the opposite direction (*87*). The IL-1β-511T allele has been reported to be less frequent in patients with Helicobacter pylori infection in early onset immune thrombocytopenic purpura (ITP) (*88*) .

1.5.3 Interleukin 6

Interleukin-6 (IL-6) is a protein produced by T-cells, macrophages, and endothelial cells. Its major function is as an acute phase protein, where it stimulates B- and T-cell growth and differentiation. IL-6 is also an important mediator of fever, and the protein can influence the effect of other cytokines. IL-6 can function both as a pro- and anti-inflammatory cytokine. As an anti-inflammatory cytokine it is inhibiting TNFα and IL-1 production, and triggering IL-1ra and IL-10 activation. The gene is located on chromosome 7, at position 7p21, and several polymorphisms have been reported. The most frequent studied genetic variation is a SNP located in the promoter region of the gene, -174G/C. Several studies have shown that this SNP regulate the production of the protein, and that the -174G allele seems to be associated with high levels of IL-6 (*89, 90*). The wild type allele in position -597 and -174 have been found associated with an unfavorable outcome of chronic hepatitis C virus (HCV) infection, in particularly in males (*91*).

1.5.4 TNFα

Tumor necrosis factor alpha (TNFα) is a transmembrane protein produced by macrophages, monocytes, neutrophils, T-cells and NK-cells as a result of the presence of bacterial toxins. Its primary function is to regulate immune cells, induce apoptotic cell death, stimulate inflammation, and control viral replication. The gene encoding TNFα is located on chromosome 6, position 6p21.3, which is a very polymorphic region of the chromosome with a cluster of immune genes, including the major histocompability complex (MCH) and complement component C4. The TNFα gene also contains a large number of polymorphisms, including -1031T/C, -857C/T, -244G/A, -308A/G and -238G/A. The -308 allele is the most studied variant, and this SNP has been found associated with a number of diseases such as rheumatic heart disease and obstructive sleep apnea syndrome (*92, 93*). The -308A allele is also reported associated with severe cerebral malaria, and with death due to meningococcal sepsis and septic shock (*94-96*).

1.5.5 Interleukin 8

Interleukin 8 (IL-8) is a chemokine produced mainly by macrophages and epithelial cells, but also by the endothelial cells were it is stored in vesicles. The main function

is to serve as a chemical signal that recruit neutrophils and induce chemotaxis in target cells, which causes the migration of neutrophil cells from the blood stream to the tissue. The gene encoding IL-8 is on chromosome 4, position 4q12-q21, and several SNPs have been reported. The most investigated SNP is -251A/T, and association between the -251AA and high IL-8 production have been reported in patients with diarrhea (*97*). Hull et al showed an association between the -251A allele and vulnerability to disease after a respiratory syncytial virus (RSV) infection (*98*), and in a Japanese study an association between -251A and gastric ulcer and gastric cancer in patients with Helicobacter pylori infection was found (*99*).

1.5.6 Interleukin 12

Interleukin 12 (IL-12) is produced by dendritic cells and phagocytes, and is a T-cell stimulating factor responsible for differentiation of T-cells. IL-12 also activates IFN-γ and TNFα production. IL-12 is able to block the formation of new blood vessels. This anti-angiogenic activity is achieved by stimulating the production of IFN-γ which in turn mediate the release of a chemokine. The two chains forming the IL-12 complex are coded by the two genes IL-12A and IL-12B, which is located at 3p12-13.2 and 5q31.1-q33.1 respectively. Several polymorphisms have been described that have functional effect in both subunits (*100, 101*). A study performed on multiple sclerosis patients suggests that IL-12 +1188A/C together with a SNP in interferon gamma at position +874A/T could be a risk factor for developing the disease (*102*).

1.5.7 Interleukin 18

Interleukin 18 (IL-18) is produced by macrophages, and induces cell mediated immunity as a response to bacterial toxins, as well as stimulates the production of IFN-γ. The gene is located on chromosome 11, and mapped to position 11q22.2-q22.3. Several SNPs have been reported, including -607C/A and -137G/C. These SNPs have been found associated with increased risk of ischemic stroke (*103*). Another study found that the -607C allele was associated with systemic lupus erythematosus, and that the C allele may result in an enhanced production of IL-18 (*104*).

1.5.8 IFN-γ

Interferon gamma (IFN-γ) is produced by natural killer cells (NK cells), and by CD4 and CD8 cytotoxic T lymphocytes. It is an important cytokine for the immunological resistance against viral and bacterial infection as well as for tumor control. However, it' most important effects are attributable to its immunostimulatory and immunomodulatory effects. The gene encoding IFN-γ is located on chromosome 12, position 12q15. Several SNPs have been reported in this gene, the most commonly investigated are -764G/C, +874A/C and -179T/G. It has been demonstrated an association between IFNγ +874A and severe acute respiratory syndrome, and it is suggested that the allele might be involved in the pathogenesis of severe acute respiratory syndrome (SARS) due its ability to alter the IFN-γ production. (*105*). The +874AA genotype have been found associated with hepatitis B (*106*), while the +874T allele is associated with symptomatic parvovirus B19 infection (*107*).

1.6 Anti-inflammatory cytokines

Anti-inflammatory cytokines are immunoregulatory cytokines that control the pro-inflammatory cytokines by reducing their production, and thus counteract their biological effect. The major anti-inflammatory cytokines are interleukin 1 receptor antagonist (IL-1ra), IL-4, IL-10, IL-11, and IL-13.

1.6.1 Interleukin 13

Interleukin 13 (IL-13) is a protein secreted by activated T lymphocytes, and IL-13 controls the functions of monocytes and B-cells. It has structural similarities with IL-4, and thus has similar functional effects and a common signaling pathway. One important role for IL-13 is to down-regulate the production of TNFα. The gene encoding for IL-13 is located in a cluster of other interleukin genes at chromosome 5, and maps to position 5q23. This gene is not as polymorph as many other interleukin genes, but some SNPs have been reported, including the haplotype variant -1112T/+2044A that have been shown to be decreased in patients with Graves' disease (*108*). Also a regulating SNP at position +4464A/G (glu110arg), has been reported, and the 110arg allele is reported associated with asthma (*109*).

1.6.2 Interleukin 16

Interleukin 16 (IL-16) is in general produced by lymphocytes and epithelial cells, and works as a chemo-attractant for CD4+ cells, monocytes and eosionphils. IL-16 can also stimulate the production of IL-2 receptors and induce HLA-DR expression. The gene encoding IL-16 is located at chromosome 15, position 15q26.1. The most common SNP is -295T/C, and it is demonstrated that the -295T allele induces a reduced promoter activity in patients with asthma (*110*). It has also been shown an association between specific variants of three SNPs, including the G allele of rs11556218, -285C and the T allele of rs4072111, and increased risk of systemic lupus erythematosus (*111*).

1.6.3 Interleukin 1ra

Interleukin l receptor antagonist (IL-1ra) is a protein which inhibits IL-1α and IL-1β by blocking the IL-1 receptors, resulting in lack of biological effects for these interleukins. IL-1ra is produced by macrophages and monocytes, and released in a much higher concentration than both IL-1α and IL-1β. The gene encoding IL-1ra is mapped to 2q13-q14.1. The most commonly investigated polymorphism in the IL-1ra gene is a VNTR in intron 2 of the gene, and it is shown that the IL-1ra allele 2 is more frequent in patients with chronic inflammatory disease as well as in autoimmune disease and solid tumors (*112*).

1.7 Genetic variations

1.7.1 Mutations

A mutation is defined as an alteration in genetic material. A point mutation is defined as the replacement of one base in DNA with another, which may have different outcome, depending on the location in the gene.

A missense mutation results in a change in the codon, and thereby to the introduction of a new amino acid in a given protein. A well known example of this is sickle cell anemia that is caused by an autosomal recessive mutation in the β-globulin gene, where an adenine (A) to thymin (T) change make an alteration in the codon from GAG to GTG, and further to an amino acid alteration, where the hydrophobic

amino acid valine replaces the hydrophilic glutamic acid at the sixth amino acid position of the polypeptide chain.

A nonsense mutation is a point mutation that changes the codon into a stop codon, resulting in a truncated protein with suboptimal function, while a silent mutation is a change that does not alter the codon, and thus does not affect the protein. An insertion or deletion mutation adds or removes segments of DNA. Such changes may include only a few base pairs, or may be more extensive losses or gain of DNA. Transposons are mobile genetic elements that can also cause mutations.

Most genetic mutations are harmful for the organism. Therefore, the cells have repair mechanisms in order to avoid accumulation of defected proteins.

1.7.2 Polymorphisms

Polymorphisms are genetic variations inherited along Mendelian lines in a family that by definition should occur at a frequency of 1% or more within a population. In genetics, polymorphisms describes the diversity within a population. Translated it means “many forms”, and polymorphisms that are conserved through generations are the evolutionary strongest genetic markers. A polymorphism may or may not have a biological effect. Single nucleotide polymorphisms (SNP) are variations in a single nucleotide at a given position in the genome. SNPs are very common, and can be observed at every 100 to 300 base along the human genome, which consists of approximately 3 billion bases. The most common variation is the replacement of thymine (T) with cytosine (C). SNPs can occur anywhere along the DNA strand, and might affect the coding or the non-coding part of a gene. Due to the stability and ability to conserve SNPs in generations, such genetic variation is an important tool for population studies.

Most SNPs have no functional effect on biological processes, even if they are located within the coding sequence of a gene. However, some of the SNPs might affect the synthesis of the given protein, and thus predispose to disease. It is important to bear in mind that a SNP does not cause disease by itself, but may be used as a marker for the probability that someone will develop a disease. An example of SNPs as markers for disease is the different allele types for apolipoprotein E (ApoE), were the allele E4 has shown to be found more often in patient that develops Alzheimer disease (*113*).

Copy number variations (CNV) are duplications or deletions of large segments of DNA, and approximately 2000 CNVs have been described in the human genome. The size of the duplicated or deleted sequence may vary from about approximately one thousand base pairs to an entire gene. CNVs that effect critical developmental genes may cause disease.

Various number of tandem repeats (VNTR) are nucleotide sequences that is repeated one after another tandemly (Figure 4). The repeated sequence may vary in length, from only a few bases to hundred or more, and the number of repeats at a given position may vary between individuals. A VNTR may influence the expression of a gene, leading to a higher or lower protein level. As for SNPs, VNTRs cannot cause disease by themselves, but may be used as markers for the risk of developing certain diseases.

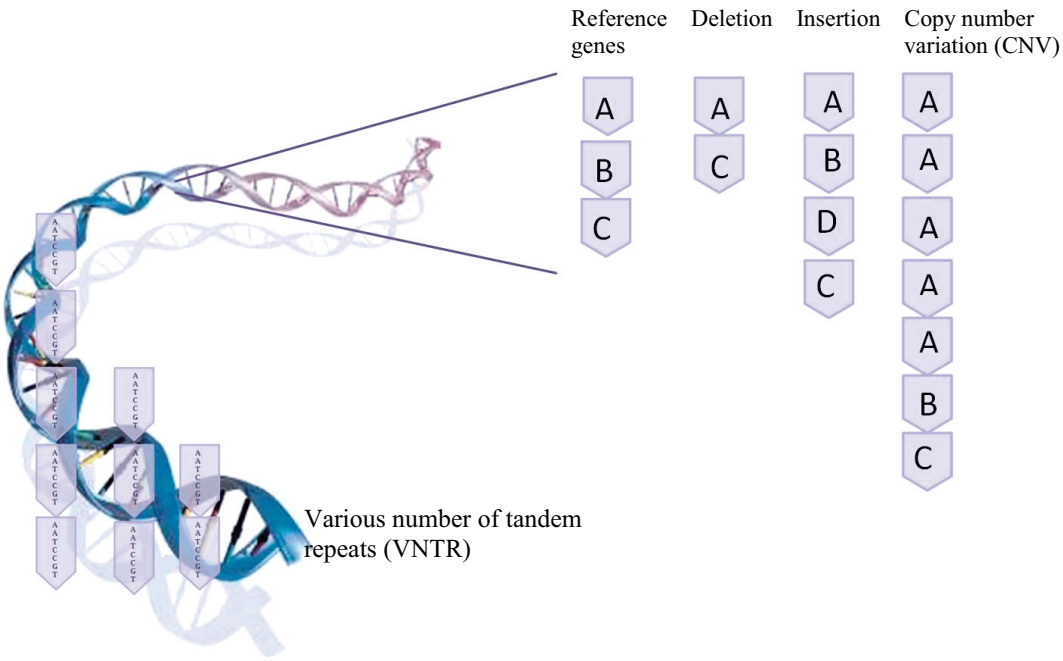


Figure 4: Illustrating four types of genetic variations; deletion, insertion, CNV and VNTR.

2 Previous studies on SIDS and polymorphisms in genes involved in the immune system

The first gene involved in the immune system to be investigated with regard to SIDS was the gene encoding complement component C4. The C4 gene was investigated in 40 German and 104 Norwegian SIDS victims, and both studies found an association between slight infection prior to death and partial deletions of the C4 gene, indicating that this combination of genetic make-up and environmental influence, may increase the risk for SIDS (*51, 52*).

The candidate genes most likely to explain the vulnerability to infection are the cytokine genes. Several studies have investigated these genes in an attempt to uncover associations between SIDS and different genetic variants (*114-119*). IL-10 is an important immune regulatory cytokine, and the SNPs in the promoter region in position -1082, -819 and -592, as well as the microsatellite IL-10R and IL-10G, are collectively responsible for the production of the protein (*120*). Both SIDS cases and cases of infectious death have been reported associated with the ATA haplotype and the ATA/ATA genotype of this gene (*114, 115*). In addition, a higher percentage of the genotypes G21/G22 and G21/G23 in cases of infectious death compared to SIDS, and a higher percentage of G21/G22 in the SIDS cases compared to controls have been reported (*114*). Based on these findings it may be speculated that in some situations an infant with an unfavorable IL-10 genotype may exhibit aberrant IL-10 production, which in turn leads to a disturbed immunological homeostasis.

The polymorphisms IL-1β -511C/T and +2018T/C, located in the IL-1Ra gene, have a significant effect on the IL-1β levels, but no association to SIDS has so far been established (*116*). Another study investigated the 89bp VNTR in the IL-1Ra gene in Australian SIDS cases, and found that carriage of the 2/2 genotype increased the risk for SIDS compared with the predominant 1/1 genotype (*121*). Homozygous carriers of allele 2 show a more severe and also prolonged proinflammatory immune response compared to other IL-1Ra genotypes (*112*), which may contribute to the vulnerability to infection seen in SIDS.

A British study including common polymorphisms in the genes encoding IL-4, IL-6, IFN-γ, TGF, and VEGF found significant differences for the genes encoding IL-6 and VEGF: the genotypes -174GG, and -1154AA were more frequent in SIDS cases than in controls (*117*). Even though only a small number of SIDS cases was

included, the authors suggest that the causation of SIDS is related to both fetal lung development and an infant`s innate ability to mount an inflammatory response to infection (*117*). The findings regarding the IL-6 gene has been confirmed in a study of Australian SIDS cases (*118*), but not in a Norwegian study (*119*).

Surfactant protein A (SP-A) and surfactant protein D (SP-D) are humoral molecules involved in the innate host defense against various bacterial and viral pathogens. Ten SNPs that might influence expression of the genes encoding these two surfactants have been investigated in SIDS cases and controls (*122*). No difference in genotype distribution was found, even though there was a tendency for the most common SP-A haplotype, 6A2/1A0, to be overrepresented in cases with low immunohistochemical SP-A expression (*122*). The SP-D expression was not influenced by any of the investigated SNPs.

An increased vulnerability to infection may also be due to genetic variation in the genes encoding G-proteins. The most investigated polymorphism in the Gβ3 gene is C825T, and it is shown that T-allele results in increased G protein mediated signal transduction compared to the C-allele (*123*). Most interleukin-receptors are G-protein coupled, and an association between the Gβ3 825T allele and increased cell function has been reported. A study of the C825T polymorphism in SIDS victims, cases of infectious death and live infant controls, revealed no difference in genotype frequency between SIDS cases and controls (*124*), but an association between the CC genotype and infectious death was found. The observation may indicate that the presence of the 825T allele exerts a protective effect towards serious infection, perhaps through enhanced G protein signaling (*124*).

3 Aims of the study

The main hypothesis of this thesis is that infants that die from SIDS, besides being at a vulnerable developmental stage, have a combination of polymorphisms in genes involved in the immune system, that predispose to disturbed homeostasis when challenged by virus or bacterial infection. Most important for such a mechanism seems to be the genes encoding the interleukins. Based on this assumption the following objectives were formulated:

1. To search for genetic predisposing factors in the gene encoding TNFα in SIDS victims and infants suffering infectious death. A SNP profile will be created with the given genotype combination of all SNPs. The aim was to disclose whether polymorphisms expressed in certain genotype combinations were associated with SIDS and infectious deaths, because possible significant associations might be camouflaged in a single SNP study.

2. To investigate functional polymorphisms in the cytokine network in a case-control study of SIDS victims, infectious deaths and adult controls. The SNPs and VNTR to be included should be selected due to their previously reported functional effect. Because all the cytokines function together in a well regulated network it would also be of interest to construct a SNP profile, to further understand the total effect. Based on the concept of the fatal triangle for SIDS (*76*) we also wanted to examine possible associations between specific interleukin genotypes and environmental risk factors for SIDS, including prone sleeping position, co-sleeping, maternal smoking, and slight infection.

3. To search for a possible association between interleukin gene polymorphisms, slight infection, and immunstimulation of the laryngeal mucosa in SIDS. By using HLA-DR expression in laryngeal mucosa as a marker of immune stimulation, the SIDS victims were classified with regard to degree of immune stimulation. To search for possible genetic risk factors for SIDS we wanted to study the genetic makeup in the interleukin genes in SIDS victims with intense and extended laryngeal HLA-DR expression compared to SIDS cases that did not show signs of such expression.

4 Material and methods

4.1 Subjects

The subjects included in the studies of this thesis were all examined at the Institute of Forensic Medicine, University of Oslo, during the period 1988-2006. The autopsies were performed as soon as possible after death and for most cases within 36 hours. Tissue samples and body fluids were collected in all cases based on a well established protocol (*26, 125-127*), and the post mortem examinations of every infant was performed by the same forensic pathologists (TOR, ÅV, MA, ASP). All individuals were Caucasians from the south-eastern part of Norway.

The subjects included 148 SIDS cases, 56 borderline SIDS cases, 41 cases of infectious death, and 131 adult diseased controls (table 2). The SIDS and borderline SIDS cases were diagnosed according to the Nordic criteria (*26*), were it is demanded that both clinical history, autopsy findings and investigation of the circumstances of death provides no explanation of death. The cases undergo a full body X-ray prior to autopsy, and before a diagnosis of SIDS or borderline SIDS can be established, toxicological screening has to be negative, and mutations and functional polymorphisms in the LQTS genes, as well as the A985G mutation in the MCAD gene, have to be excluded. Afterwards, an informed consent questionnaire was send to the SIDS parents to obtain knowledge of the history of the child's health in the days before death, parental smoking, position when found dead and sleeping environment. All subjects included in the group of infectious death died suddenly and unexpectedly, without a history of severe illness prior to death. The causes of death in the control group were acute non-infectious disease, trauma, and intoxication.

Table 2: Survey of the patients

Group	Number of cases	Diagnosis	Gender (F/M)	Age at time of death, median (range)
SIDS	148	SIDS	57/91	3.8 mo (0.1-24 mo)
	56	Borderline SIDS	20/36	3.5 mo (0.5-39 mo)
Infectious death	41	19 pneumonia 16 septicaemia 1 meningitis 2 CMV infection 1 encephalitis 1 myocarditis 1 peritonitis	13/28	7.6 mo (0.8-39 mo)
Controls	131	52 disease 23 intoxication 56 violent death	43/88	49 y (11-86 yrs)

4.2 Bioinformatics

In the papers included in this thesis, a total of 14 SNPs were investigated using MassArray technology. As a first step, the genetic variation in the sequences surrounding each SNP has to be identified. The surrounding sequence may be obtained from the SNP-database, dbSNP, at NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP/). In order to use this database the reference SNP (rs) or the submitted SNP (ss) number for the SNP has to be known.

A typical input file should contain about 250 bases up- and down-stream from the SNP of interest, and the next step is to localize and mark all genetic variations in this approximately 500 bp sequence. This can be done by SNP Basic Local Alignment Search Tool, or SNP-BLAST, at UCSC Genome Browser website (http://www.genome.ucsc.edu/). At this web site the DNA sequence may also be extended, if that obtained from dSNP is not sufficient. This may happen if the sequence obtained from the dbSNP is short, or if the sequence is very polymorphic, making it difficult to design primers.

In cases were the rs or ss number was not known, the DNA strand was obtained from the UCSC Genome Browser by first obtaining knowledge about the primers previously used to genotype the SNP, then blasting the reverse and forward primers. This will provide the DNA sequence including the SNP of interest. However, the product obtained from this procedure will most often not be sufficient to obtain all the information necessary to do a multiplex priming. Therefore it may be necessary to perform a new blast to obtain a prolonged sequence on both sides.

When both the SNP of interest, as well as other SNPs in the surrounding sequence, have been marked, the sequence have to be repeatmasked. RepeatMasker is a program that screens DNA sequences for interspersed repeats and low complexity DNA sequences. Approximately half of the human genomic DNA will be masked using the repeatmask programs. These programs are useful for analytical purposes using sensitive technology in which interspersed repeats must be known. Several programs are available for this task, and the most conservative repeat masking web site is the SNPmasker 1 (http://bioinfo.ut.ee/snpmasker/). The one used for most of the SNPs in the papers included in this thesis, is the program found at the website http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker.

4.3 DNA extraction

DNA was extracted from spleen using standard methods for robot extraction and the BioRobot EZ1 (Qiagene, qiAmp DNA minikit). The robot extractor uses magnetic particles technology, where DNA binds to the silica surface of the magnetic particles. All reagents are supplied in a kit and are prefilled into cartridges, which ensure a sterile environment for the extraction process. The EZ1 provides very pure DNA, which is important since only a small amount of DNA are used in the further analytical process. DNA from some of the oldest cases was extracted from blood or spleen either in a 340A Nucleic Extractor (Applied Biosystem, Rotterdam, Nederland) or manually, both using standard methods (phenol-chloroform extraction and ethanol precipitation) (*128*). Though the phenol-chloroform and ethanol precipitation does yield a higher DNA concentration, the process is time consuming and it requires a lot of on-hand laboratory work. The purity of the DNA is not as good as the DNA obtained from the BioRobot EZ1.

4.4 *DNA quantification*

The samples were quantified using realtime PCR (qPCR) and a real time PCR machine Mx3000p (Stratagene). The quantification kit used for the analysis was Quantifiler (Applied Biosystems).

When quantifying DNA using a qPCR quantification method, the target concentration is calculated as a function of PCR cycle number. The chemistry used involves, like conventional PCR, dNTP, primers and polymerase, and the increase of amplified products are measured as an accumulation of fluorescence after each PCR cycle. The concentration of DNA in the sample is inversely proportional to the cycle threshold (ct value), which is the number of cycles needed for the measured fluorescent signal to pass the background noise and exceed the threshold. The threshold is set to the exponential phase were the reading would be most accurate. A real time reading is commonly plotted into a diagram, and the curve will most often have a sigmodial shape (figure 5) with the copy number on the Y-axis and the cycle number on the X-axis. In the exponential phase the number of DNA strands double for every cycle, and a plateau phase is reached when there is no more available primers in the mastermix. After quantification, all samples were diluted to a concentration of 20 ng/µl.

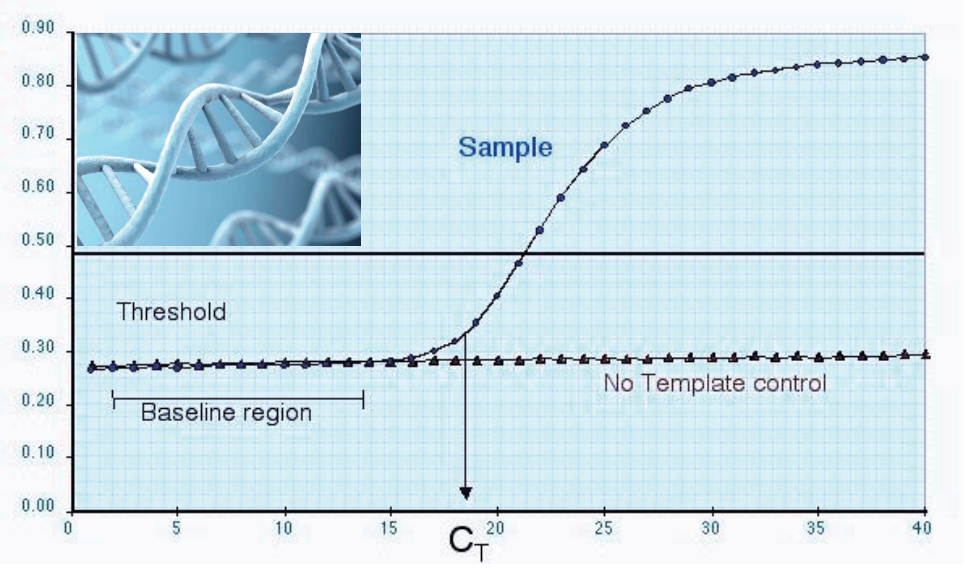


Figure 5: Real-time PCR image with a steady exponential phase for the sample.

4.5 Sequenom® multiplex

The SNPs included in table 3 and figure 6 were genotyped using MassARRAY™ on a SEQUENOM® platform, in cooperation with CIGENE, Norwegian University of Life science, Ås. As core facility under the Norwegian Functional Genomics program (FUGE), CIGENE is a national platform for SNP analysis. The total process is automated using highly specialized instruments such as Beckman Biomek Fx pipetting robot (Beckman Coulter), a Beckman Multimek96 pipetting robot (Beckman Coulter), a nano-dispenser chip-printing robot, a mass spectrometer, and a Laboratory Information Management System (LIMS system).

A multiplex assay was designed using Assay designer software 2.0 (SEQUENOM® Europe) that automatically design primers. Following polymerase chain reaction (PCR) and Shrimp Alkaline Phosphatase (SAP) treatment, a primer extension reaction was performed to introduce mass-differences between alleles and product, the reaction was further desalted using resin. The PCR product was thereafter dispensed onto a SpectroCHIP with 384 patches. Approximately 25nl amplicon was transferred onto the pins in the silica chip. The mass difference were detected using a matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) and genotypes were called using type 3.1 software (SEQUENOM®). The energy from the laser is absorbed by the matrix, which is an important step that will cause part of the illuminated substrate to vaporize. Once the laser beam have caused vaporization and ionization of the molecules, it is transpired electrostaticly into the vacuum tube were the mass-to-charge (m/z) is detected. The time flight for the ion in the tube is proportional to the square root of its m/z. Cluster plots were made due to the signals of different allele mass. In order to avoid contamination the pre- and post-PCR procedures were kept geographically separated, and the sample plates had a one way work flow. The gene sequences were obtained from SNP database build 127 (http://www.ncbi.nlm.nih.gov/sites/entrez)

Table 3: Functional SNPs investigated using MassArray

Gene	Polymorphism	rs number
IL-1α	+4845G/T	rs17561
IL-1β	-511C/T	rs16944
IL-6	-572G/C	rs1800796
IL-8	-781C/T	rs2227306
	-251A/T	rs4073
IL-12	+1188A/C	rs3212227
IL-13	+4464 A/G	rs20541
IL-16	-295T/C	rs4778889
IL-18	-137G/C	rs187238
TNF-α	-1031C/T	rs1799964
	-857 T/C	rs2507961
	-244A/G,	rs673
	-238 A/G	rs361525
IFN-γ	+874A/T	rs2430561

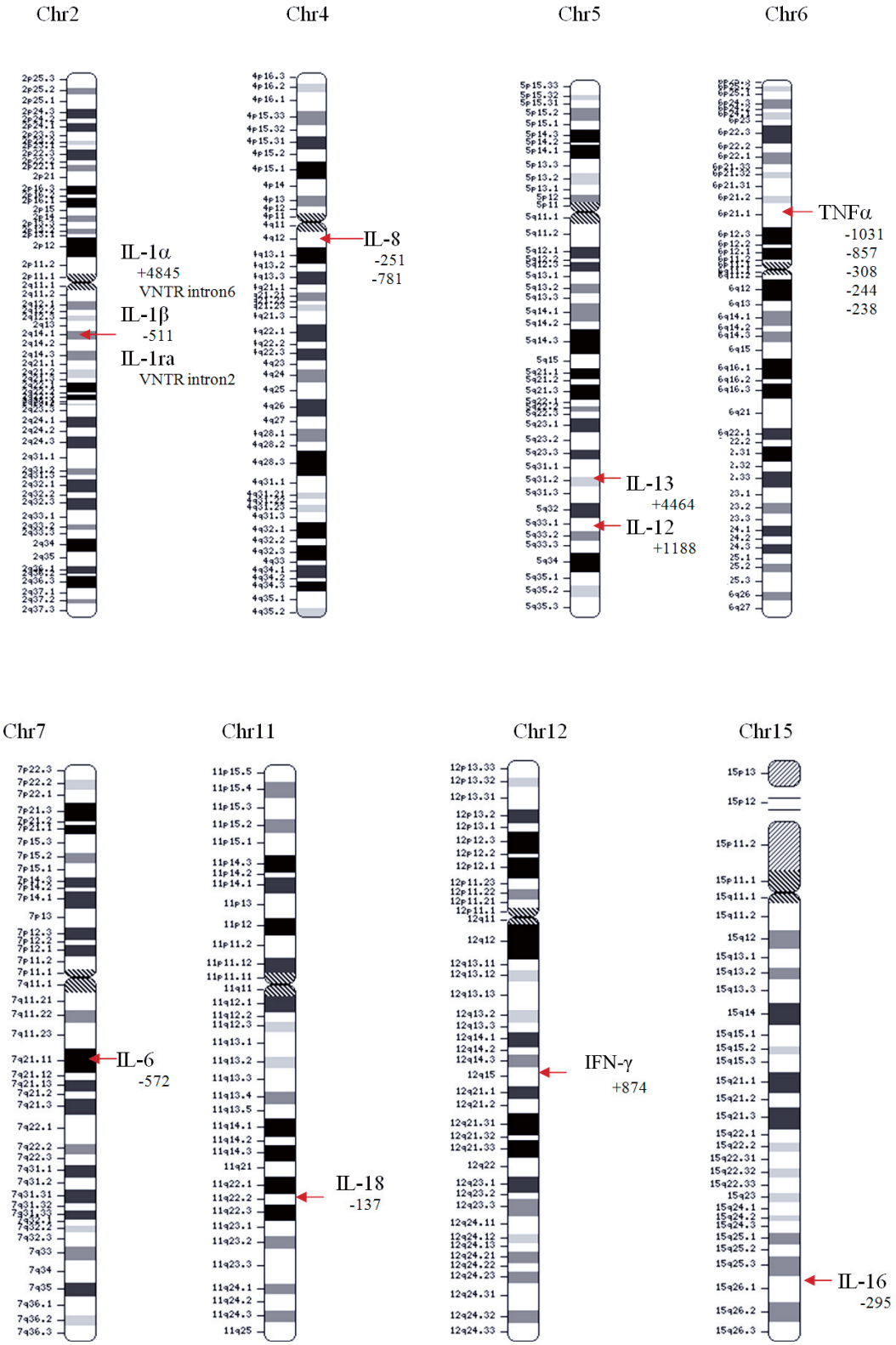


Figure 6: Chromosomal localization of the polymorphisms studied in the present thesis.

4.6 *TaqMan genotyping*

One of the SNPs in the TNFα gene, -1031C/T, was analyzed using TaqMan SNP genotyping assay (Applied Biosystems) and Mx3000p real-time PCR machine (Stratagene, a Jolla, CA). The assay id was C_7514871_10. The TaqMan genotyping assay is based on allelic discrimination using two allele-specific fluorescent probes together with primers flanking the sequence of the SNP. The probes have a quencher at the 3' end to avoid fluorescence signal. The two probes are marked with different reporter fluorescence at the 5' end, both specific to one of the alleles. When the target specific probe anneal to the sequence, the quencher is released from the probe and the fluorescence can be measured (Figure 7).

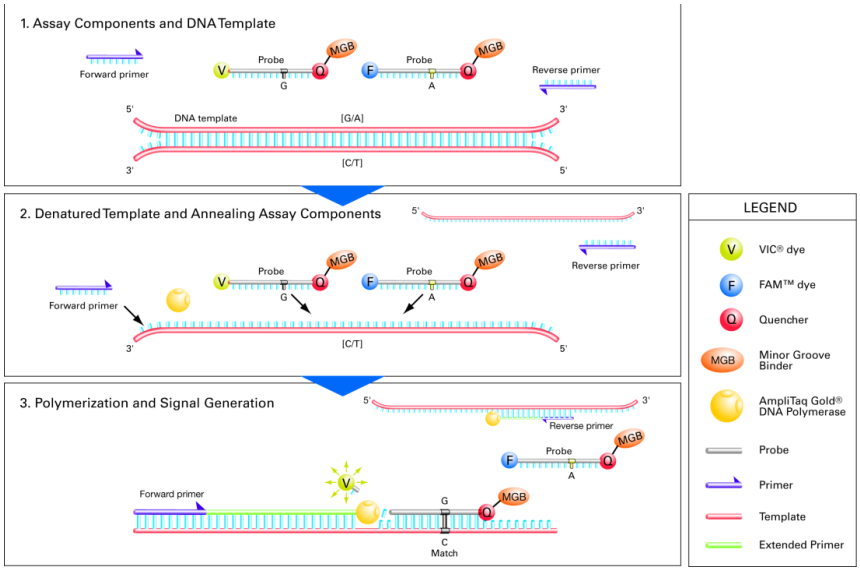


Figure 7: Allelic discrimination by TaqMan probes. The probes are labeled with the fluorophore VIC or FAM. These are attached to the probes, and the quencher prevents liberation of the reporter fluorescence if the probe is not degraded. The Minor Groove Binder (MGB) function as a stabilizer for the double-stranded structure formed between the target and the probe. Probes that hybridize specifically to DNA fragments are destroyed and the fluorescence of corresponding fluorophore is liberated and creating a signal. (Source Applied Biosystems).

4.7 Sequencing

DNA sequencing is an analytical method that determines the exact nucleotide (adenin, thymin, cytocine and guanine) order in a given DNA strand. One sequencing method is the dideoxy metode that synthesizes DNA from four deoxynucleotide triphosphates (dTTP). This is achieved by adding a new nucleotide to the 3' –OH group at the growing DNA strand. The elongating is terminated when there is no 3' –OH group attached to the last nucleotide. The reaction mixture contains all four normal nucleotides (dNTP) and the dideoxynucleotides (ddNTP), the latter in limited concentration. All are labeled with a different fluorescence color. When the elongation have been terminated, the fragments are separated based on their length, and the fluorescence from the different dideoxynucleotides are visualized by laser.

4.8 Construction of SNP profiles and VNTR/SNP combinations

In paper I, a TNFα SNP profile was made for 135 of the SIDS cases, 56 of the borderline SIDS cases, 38 of the cases of infectious death and 126 controls. This was done by assigning the genotypes of every SNP with a number from 1-3, were the most common homozygote was 1, heterozygote 2, and homozygote for the other allele 3. The genotype combinations for all cases were plotted manually. Only the cases with results in all SNPs were included. The SNP -244 was excluded from the profile construction as it was found to be heterogeneous in the population.

In paper II, a SNP profile was constructed for the pro- (IL-6, IL-8, IL-12, IL-18 and IFN-γ) and anti- inflammatory interleukins (IL-13 and IL-16) separately, and also for interleukins located on the same chromosome. This was performed by clustering the SNPs belonging to the appropriate group.

In paper III, a combined SNP/VNTR gene combination was constructed in 135 SIDS cases, 56 borderline SIDS cases, 38 cases of infectious death and 126 controls.

4.9 Allele specific PCR

The TNFα-308G/A (rs1800629) polymorphism was genotyped using allele specific PCR. The PCR reaction was performed using standard protocols for normal amplification. Each sample was included in two PCR reactions with different sequence specific primers, the primer pairs used together were TNF-α1 / TNF-α2 and

TNF-α1 / TNF-α3 (table 4). In addition, the PCR reactions included internal control primers, IK1 and IK2, to avoid false negative results (*129*). The PCR conditions were 95 ^{o}C for 30s, 64 ^{o}C for 30s, and 72 ^{o}C for 30s, 30 cycles (*129*).

Table 4: Primer sequences for allele specific genotyping of TNFα -308 A/G.

Area	Primer Sequence
-308G	TNF-α1: 5'-CTCGGTTTCTTCTCCATCG-3',
	TNF-α2: 5'-ATAGGTTTTGAGGGGCATGG-3',
-308A	TNF-α1: 5'-CTCGGTTTCTTCTCCATCG-3'
	TNF α3: 5'-AATAGGTTTTGAGGGGCATGA-3'
Human	IK1, 5'-GCCTTCCCAACCATTCCCTTA-3'
Growth	IK2, 5'-TCACGGATTTCTGTTGTGTTTC-3'
Hormone	

4.10 VNTR analyses

Two VNTRs were analyzed in the papers included in this thesis, a VNTR in intron 2 of the IL-1ra gene and a VNTR in intron 6 of the IL-1α gene. The polymerase chain reaction was performed using standard protocols for normal amplification. Primers for both VNTRs were designed using the program Primer3 (http://frodo.wi.mit.edu). The sequences of the primers flanking the IL-1α VNTR were 5'-GCCTCTAGACTCATAGAACTTAGTC-3', and 5'-GTGAGGTCAGGCCATTGCACTG-3'. The temperature profile was 96 ^{o}C for 1 min, 58 ^{o}C for 1 min, and 74 ^{o}C for 6 min, for 30 cycles, followed by a final extension at 72 ^{o}C for 10 min. The sequences of the primers flanking the IL-1ra VNTR were 5'-CTCAGCAACACTCCTAT-3' and 5'-TCCTGGTCTGCAGGTAA-3'. The temperature profile was 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, for 30 cycles, followed by a 10 min final extension at 72°C.

4.11 Gel electrophoresis

Amplified products were detected by gel electrophoresis on 2% agarose gels in the presence of ethidium bromide, and the DNA bands were visualized using ultra violet (UV) light. The PCR products of TNFα were 185 bp of size, the IK product were 427 bp. The alleles of the VNTR in intron 6 of the IL-1α gene ranged from 620 bp to 1220 bp, while the alleles of the VNTR in intron 2 of the IL-1ra gene ranged from 240 bp to 595 bp.

4.12 Immunohistochemistry, HLA-DR

Tissue sections from laryngeal mucosa were stained using immunohistochemistry. The primary antibody used was a mouse anti-human HLA-DR monoclonal antibody which were incubated with components of the Envision detection system peroxidase/DAB+ labeled rabbit anti mouse (EnVision+/HRP/Mo, Dako, Glostrup, Denmark). Negative controls were treated identically, except that the primary antibodies were omitted.

To evaluate the HLD-DR expression in mucosal glands and surface epithelium scores were given according to a previously reported protocol, with slight modification (*66*). Four variables were evaluated in each tissue section: staining intensity and extensiveness of positive staining of both surface epithelium and mucosal glands. Intense positive staining in surface epithelium (squamous and columnar) and glands was given a score of 2, intermediate staining was scored 1, and no staining in the surface epithelium and glands was scored 0. As to the extensiveness of staining, more than 50% of the cells was scored 2, staining up to 50% of the cells was scored 1, and no staining was scored 0. The slides were evaluated using a LeicaDMLB microscope (Leica Microscopie and system GmbH, Wetzlar, Germany) equipped with 10x magnification of the ocular lens and 40x magnification of the objective. Images of the slides were taken with a Leica digital camera.

Blind inter – and intra observer reproducibility testing of the scoring of intensity and extensiveness of staining was performed involving two observers (LF, ÅV).

4.13 Statistical analyses

The χ^2 – test was used for comparing the frequencies of the different polymorphisms between the diagnostic groups, as well as to correlate genotypes with external risk factors for SIDS. When cell count was low (<5), the Monte Carlo test was applied. For testing of inter- and intra-observer reproducibility regarding scoring of HLA-DR expression, the Kappa test was performed. The level of significance was set to 0.05. When investigating the relationship between interleukin levels in the CSF and HLA-DR expression, the Kruskal-Wallis test was used. Calculations for paper I was performed using SPSS version 14.0, for the other papers SPSS version 16.0 was used (SPSS, Chicago, IL, USA).

Hardy Weinberg equilibrium was calculated using OEGE - Online Encyclopedia for Genetic Epidemiology studies.

4.14 Registry and approval

All the studies were approved by the Committee for Medical Research Ethics in Southern Norway (REK sør, ref nr S-06264a).

5 Summary of papers

5.1 Paper I

Ferrante L, Opdal SH, Vege Å, Rognum TO. **TNF-α promoter polymorphisms in sudden infant death.** Hum Immunol 2008;69:368-73.

Several studies have suggested that there is an association between SIDS and an activated immune system. Tumor necrosis factor (TNFα) is a multifunctional pro-inflammatory cytokine, produced by mainly macrophages and monocytes, that is important in many biological processes during the immunological response . The TNFα gene is located at chromosome 6 in-between a cluster of other immunological genes, a location known to be highly polymorphic. The aim of the study was to investigate functional polymorphisms in the promoter region of the TNFα gene in order to investigate any possible associations between TNFα genotype and SIDS. SNPs at the position -1031T/C, -857C/T, -308G/A, -244G/A, and -238G/A were included. The subjects investigated in the study include 148 SIDS cases, 56 borderline SIDS cases, 41 cases of infectious death, and 131 adult controls.

Results: When investigating each SNP separately, associations between -238GG and SIDS (p=0.022) and between -308GA and borderline SIDS (p=0.005) were found. There were no associations between any of the other SNPs investigated. Furthermore, a SNP profile was constructed for each case by creating a genotype pattern from the investigated SNPs. Fifteen gene combinations were obtained, and four profiles had significantly different frequencies in SIDS cases and controls. The two SNP profiles -1031CT, -238GG, -857CC, -308GG and -1031TT, -238GG, -857CC, -308AA were found significantly more often in SIDS than in controls, and may thus be unfavorable.

Conclusions: The findings of the study add evidence to the theory that an unfavorable genetic profile in the TNFα gene may be involved in SIDS. Both high levels and prolonged exposure to TNFα, combined with reduced release of anti-inflammatory cytokines, might cause a deleterious immunological imbalance in an infant at vulnerable developmental stage of the central nervous system and the mucosal immune system, and thus contribute to SIDS.

5.2 Paper II

Ferrante L, Opdal SH, Vege Å, Rognum T. **Cytokine gene polymorphisms and sudden infant death syndrome.** Acta Paediatr 2010;99:384-8.

Increased expression of the components of the mucosal immune system in SIDS has been reported in several studies. An unfavorable genetic predisposition in genes that regulate that immune response may be the cause. In this study functional SNPs in the genes encoding IL-6, IL-8, IL-12, IL-13, IL-16, IL-18 and IFN-γ were investigated in 148 SIDS cases, 56 borderline SIDS cases, 41 cases of infectious death and 131 controls.

Results: Regarding genotype distribution, no differences between the investigated groups were found. However, in the SIDS group, the genotypes IL-8 -251AA/AT and IL-8 -781CT/TT were significantly more frequent in SIDS cases found dead in a prone sleeping position, compared with SIDS cases found dead in other sleeping positions. In addition, there was an association between fever prior to death and the genotype IL-13 +4464GG in the cases of infectious death.

Conclusion: The result from this study does support the hypotheses that specific cytokine genotypes are a part of genetic make-up that make infants sleep prone at risk for SIDS. The study supports the view that SIDS is multifactorial, including both environmental risk factors and a predisposing inheritance pattern.

5.3 Paper III

Ferrante L, Opdal SH, Vege Å, Rognum TO. **IL-1 gene cluster polymorphisms and sudden infant death syndrome.** Hum Immunol 2010; 71:402-6.

Interleukin 1 (IL-1) is a pro-inflammatory cytokine that is of great importance for the organism to fight an infection. The three subgroups (IL-1α, IL-1β, and IL-1ra) of the cytokine are enabling transmigration of leukocytes to locations were they are needed. They also induce fever by re-setting the thermoregulation center. The aim of the present study was to investigate two polymorphisms in the IL-1α gene; a variable number of tandem repeat (VNTR) in intron 6 and a single nucleotide polymorphism in +4845G/T, as well as the -511C/T polymorphism in the gene encoding IL-1β, and a VNTR in intron 2 of the competitive antagonist IL-1Ra, in 148 SIDS cases, 56 borderline SIDS cases, 41 cases of infectious death, and 131 controls. The genetic findings in all cases were also related to the environmental risk factors for SIDS.

Results: When investigating each polymorphism separately, no differences in genotype distribution between the diagnosis groups and controls were found. However, when combining VNTR and SNP genotypes, an association between the gene combination IL-1α VNTR A1A1/IL-1α +4845TT and SIDS was disclosed ($p < 0.01$). In the SIDS group it was also found that the genotypes IL-1β -511CC/CT were significantly more frequent in the SIDS victims found dead in a prone sleeping position, compared with SIDS victims found dead in other sleeping positions ($p = 0.004$).

Conclusions: The findings in the present study indicate that specific interleukin gene variants may be a predisposing factor for sudden unexpected infant death. A specific VNTR/SNP combination was found more often in SIDS cases compared to controls, and one may speculate that this genotype combination may cause an elevated expression of IL-1 that might be unfavorable.

5.4 Paper IV

Ferrante L, Opdal SH, Vege Å, Rognum TO. **Is there any correlation between HLA-DR expression in laryngeal mucosa and interleukin gene variation in sudden infant death syndrome?** Submitted

Abstract:

The mucosal immune system and the interleukin cascade are activated in a large proportion of SIDS cases, and SIDS victims with high IL-6 levels in the cerebrospinal fluid (CSF) have had clinical signs of a cold prior to death and show intense HLA-DR expression in laryngeal mucosa. It is thus hypothesised that SIDS might be due to an uncontrolled cytokine storm induced by slight infection, and that a certain genetic make-up pre dispose to such overreaction. In this paper, the intensity of HLA-DR expression in laryngeal mucosa in SIDS was studied and related to cytokine gene polymorphisms as well as CSF levels of IL-1α, IL-1β, IL-6, IL-10 and TNFα. Laryngeal sections from 97 SIDS cases were stained with a mouse antihuman HLA-DR monoclonal antibody, and HLA-DR expression (intensity and extensiveness of the staining) was scored according to a semiquantitative scoring system.

Results: In 7% of the SIDS victims intense HLA-DR expression in the laryngeal mucosa was seen, whereas 30% were negative. HLA-DR expression was compared with polymorphisms previously published for the same SIDS population. Ninety-six percent of the cases with little or no HLA-DR expression had the two most common IL-1α intron 6 VNTR genotypes A1A1 and A1A2, while 67% of the cases in with intense and extended HLA-DR were observed to have the less frequent genotypes (A2A2, A3A3, A1A3, A1A4, A3A4) ($p=0.04$). Also, IL-6 levels in CFS were found to be elevated in infants with high HLA-DR score, whereas a tendency to an inverse relationship was seen for IL-1α.

Conclusion: The results support the hypothesis of a fatal triangle in SIDS. During the vulnerable developmental period certain cytokine variants may predispose to an immunological overreaction to an otherwise harmless event, e.g. a common cold.

6 General discussion

The four papers included in this thesis are the results of genetic studies of functional polymorphisms in cytokine genes and their possible associations with characteristic features found in SIDS victims and cases of infectious death. The studies also relates the different genetic patterns to known environmental risk factors for SIDS. The most important aim of this thesis is to search for predisposing factors according to the hypothesis of the fatal triangle for SIDS (*76, 130, 131*).

6.1 Selection of genetic variations

Several studies have investigated genetic variants in genes involved in the immune system in SIDS cases (*50, 119, 122, 132*). The focus of this thesis is the cytokine network and genetic variations in cytokine genes. Polymorphisms known to affect gene expression and to be associated with various infectious diseases were selected (*86, 90, 101, 133-139*). The investigated polymorphisms are all normal gene variants, and thus not the cause of death by themselves.

6.2 Methodological challenges

There are numerous high throughput SNP genotype technologies available, and each study design will have its very own requirements to the combination of technology, accuracy, high-throughput, low-cost identification of genetic variations. The Sequenom MassArray platform provides the user with accurate and cost effective genotyping accomplished by a homogeneous reaction format, where allele-specific products with distinct masses are produced by single extension primers. Furthermore, multiplexed PCR reactions, a single termination mix and universal reaction conditions for all SNPs, small reagent volumes, and a high throughput gives high quality genotyping results.

The technology provides an automated software that designs primers for the multiplex assay. The program is designed to avoid any primer combinations that could provide complications in the reaction mix, such as nonspecific extensions. The considered software have shown the ability to primer design for more than 95% of the SNPs reported and registered on dSNP. The result of poor primer design could be

false positive results. To avoid this the platform offers a RealSNP software that are developed to scan the sequence for the possibility of any unintended products.

The method have several pre-analytical steps that need critical considerations. A conservative repeat masking may introduce some challenges in primer design process, if the sequence is masked too heavily the possibility to design a steady primer would be limited. However, there are ways to get around analytical challenges in the primer design programs, so that the SNPs may be multiplexed despite heavy repeat mask.

Another pitfall concerning the Sequenom platform is the possibility that one or more of the SNPs in a multiplex does not produce results of sufficient quality. One possibility is to re-run the chip, this is however time consuming and will result in an unnecessary additional high cost in relation to the obtained analytical data.

Should it not be possible to design appropriate primers for a multiplex analysis, alternative analytical procedures, such as TaqMan technology, allele specific PCR, and restriction fragment analysis, may be considered in order to obtain genotyping. TaqMan technology was chosen in this study for the SNP TNFα -1031 (rs 1799964), which was surrounded by a DNA sequence of such a character that a multiplex MassArray was not possible. TaqMan technology have been developed by Applied Biosystems (ABI) and allows detection of SNPs with high accuracy. The TaqMan probes are a based on the nuclease activity of the 5'-3' of the enzyme Taq-polymerase and fluorophore-based detection.

The TaqMan assay is a robust and high throughput analytical tool, however there are also some challenges that need to be addressed. The sample might fail to cluster with one specific allele. One likely explanation for this could be that the reporter dye were not assigned correctly. This will require a reset of the dye settings and reanalyzing of the plate. If one of the samples has a different DNA concentration than the rest of the samples in the same plate, problems can occur with assigning a cluster. Due to this sensitivity of the method one has to make sure that all the samples are quantified accurately and diluted to similar concentration. Another commonly occurring problem is the presence of bubbles in the wells. These are easily discovered during the last step of sample set up and avoided by centrifuging the plates. If a sample does not contain the correct reaction volume, which may happen during pre-PCR, this might also give rise to an analytical problems. Should a sample not generate a fluorescence signal, this may be due to several factors. The sample may

not contain any DNA, which can be tested by quantifying the sample. Furthermore, the sample may contain an inhibitor, or the absence of the signal could be due to rare allelic variants. In the latter case the sample must be rerun using a different genetic analytical method.

Another alternative approach is to sequence the DNA fragment that contains the SNP of interest. This may be done to verify the genotype if high quality results are not achieved by other analytical methods. However, direct sequencing is both a time consuming and a relatively expensive analytical method, but it confirms the true genotype with a high degree of accuracy. In the present thesis, direct sequencing was used to confirm the genotyping of TNFα -244 A/G (rs673). Several other genotyping techniques could have been used, such as molecular beacon, and scorpion assay which are both plate based PCR methods, as well as pyrosequencing or chip based technology. Sequencing was chosen due to the highly polymorphic sequences surrounding the SNP TNFα -244, making it an analytical challenge to design primers that would be stable enough.

Several large genome-wide association studies (GWAS) have been performed on subjects with different diseases, to reveal genetic associations that are difficult to uncover with other analytical approaches. The reason is probably due to the fact that in several diseases, inheritance or predisposition may be polygenetic, and thus be caused by many different genetic variations in concert. To perform genome wide association studies on SIDS cases does introduce several challenges. The SIDS population has to be very large in order to obtain reliable data. To our knowledge such a large sample group does not exist as one pure population. One possible approach would be to pool different SIDS populations into one large study-population, and by doing so, expanding the number of cases quite drastically. However, this approach does inflict new challenges as there most likely will be different ethnicities in such a pooled multifactorial population. This is a major problem, since most gene variants differ in frequency between different ethnic populations.

Another challenge is that the diagnostic criteria applied in SIDS diagnostic may vary considerably between countries and even between different forensic pathologists. Blind reevaluation of cases diagnosed as SIDS has disclosed striking discrepancies between the original diagnosis and reevaluated diagnoses (*58, 140*). Several researchers and forensic pathologists have put a lot of effort into reaching a

consensus on definition and diagnostic criteria (*9, 56, 141, 142*). Participants of international workshops and symposiums have discussed the diagnostic criteria in order to reach an agreement (*26, 125*), but have still not completely succeeded. These circumstances make it necessary to raise critical questions towards multinational consortia performing GWAS in SIDS.

In the present thesis SIDS case, borderline SIDS cases and cases of infectious death were obtained from the SIDS biobank of the Institute of Forensic Medicine, University of Oslo. This biobank contains samples gathered and stored since 1984. The diagnosis are based on the Nordic criteria for SIDS (*26, 58*). The adult controls were taken from the approved control biobank.

Most of the samples gave good analytical results. From evaluation of the raw data of the MassArray it is clear that the SNP genotyping has in general been a success. The results indicate a robust SNP assay and good quality DNA samples.

6.3 Multiple comparisons

Many genetic studies, especially genome wide association studies, generate several apparently significant p-values, and the interpretation of the results is thus a challenge. Tests of significance most often operate with a 0.05 level, which is a level of significance accepted by the scientific community. This imply that the probability of observing the results when there is no effect, that is to falsely reject the null hypothesis, is less than 5%. When making many calculations on the same dataset one would expect to obtain significant p-values just by chance in one out of twenty comparisons. Consequently, when the result is based upon many calculations the interpretation should be made with caution. The p-values reported in the papers included in this thesis were a result of testing hypotheses with regard to possible relationships between different genotypes, cause of death, and environmental risk factors for SIDS.

There are different methods for adjusting for multiple comparisons, and some are more conservative than others. The most commonly used is the Bonferroni correction (*143*). This test simply multiply the p-values by the number of comparisons. For a small number of comparisons (up to five) it's use is reasonable, but for larger numbers it is highly conservative. An alternative to Bonferroni test is the False Discovery Rate (FDR) method, which estimates how many of the nominally

significant associations that represent true findings (*144*). This method does not rely on cutoff-values, but estimates how many of the association tests reaching a given significance level are expected to be false. Other alternatives are Newman-Keuls, Duncan and Scheffe. Each method is aimed at controlling the overall Type I error rate at no more than 5%. The disadvantage of all these methods is that they are conservative, in that they err on the side of safety (non-significance) (*145*).

In this thesis we have applied the Monte Carlo correction in comparisons that involved a large number of small groups, and the limit of the significance was set to 0.025. Apart from that, we have not done any adjustments for multiple comparisons. Since we have tested selected genes, based on knowledge of their function, such an approach may lead to fewer errors of interpretations (*146*).

6.4 Hardy Weinberg equilibrium

The Hardy Weinberg principle claims that the genotype and allelic frequencies in a population remains in equilibrium. To meet the requirements for the Hardy Weinberg equilibrium one has to assume random mating, suppose that mutations does not occur, and that there is no migration of alleles between populations. The equilibrium can be disturbed by natural selection, genetic drift and genetic flow. A population is in Hardy Weinberg equilibrium (HWE) when it meets all these requirements, however the equilibrium is impossible in nature, as it describes an idealized state. The HWE is given as the following equation : $p^2 + 2\,pq + q^2 = 1$, were p is the frequencie of the A allele in a population and q is the frequencie of the a allele. In order to verify the quality of the control groups, a HWE test may be performed. The Hardy-Weinberg equilibrium test on the SNPs included in this thesis was performed using a web-based Hardy-Weinberg equilibrium calculator (www.oege/org/software/hwe-mr-calc.shtml) (*147*). All the SNPs included in this thesis was found to be in Hardy Weinberg equilibrium in the controls.

6.5 Inter- and intraobserver variation in the histopathological evaluation

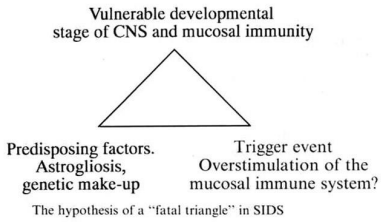
Evaluation of histological and immunohistochemical features are by nature subjective. In scientific investigation it is therefore necessary to perform inter- and

intra- observer variation testing. To be able to assess interobserver agreement in scoring HLA-DR expression in laryngeal mucosa, the histological slides of 97 SIDS cases were evaluated independently by the research fellow (LF) and by a forensic pathologist (ÅV) with experience in clinical pathology and medical microbiology. The identity of the histological sections was blinded for both the research fellow and the forensic pathologists. The scoring categories was agreed upon prior to the evaluation. The intraobserver agreement was assessed by the research fellow (LF) on blinded sections 3 months after the first evaluation. The calculated kappa value was well within satisfactory limits. Modern scientific histological and immunohistochemical studies require testing of inter- and intra- observer reproducibility and reproducibility studies are now mandatory (*148*).

6.6 *The fatal triangle*

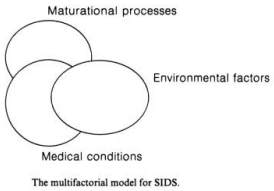
Different models (figure 8) have been put forward to explain the mechanism leading to SIDS. Valdes-Dapena proposed SIDS as a bi-phasic phenomenon. Before birth the infant is subjected to a variety of adverse intrauterine influence e.g. maternal smoking. After birth the infant is challenged by some external or internal factors or "trigger" events which he or she cannot cope, for instance an upper respiratory infection (*149*). However, already in 1972 Wegewood had proposed a triple risk model for SIDS (*150*). The models referred to in this thesis; the fatal triangle proposed by Rognum and Saugestad (*76*), as well as the triple risk model of Filiano and Kinney (*131*), and the multifactorial model of Kahn (*151*), are essentially the same. The models suggest firstly that the infant is in a particular vulnerable developmental stage during the first months after birth when the central nervous system, as well as the immune system, undergo rapid development. Secondly, there are predisposing factors such as an unfortunate "genetic make up" or scares in the brainstem. The last corner in the triangle are trigger event such as slight infections, prone sleeping, maternal smoking, and overheating, either separately or in combination.

The tripple risk model for SIDS
Filiano JJ, Kinney HC, Biology of the Neonate 1994; 65: 194-197



The hypothesis of a "fatal triangle" in SIDS

Rognum TO, Saugstad OD. Acta Pædiatr Suppl 1993; 389: 82-85



The multifactorial model for SIDS.

Kahn A Groswasser J, Kelmanson I. In: Sudden Infant Death Syndrome. New Trends in the Nineties. Ed TO Rognum. Scandinavian University Press 1995: pp 132-137

Figure 8: The different versions of the triple risk model for SIDS (76, 131, 151).

The vulnerable developmental stage. Three months after birth primitive reflexes such as turning the face away if mouth and nose are covered, have disappeared, and the infant has not yet developed experience to react rationally to such obstacles of the upper airways. This "reflex" chaos may constitute increased vulnerability. Furthermore, during the past months after birth the mucosal immune system undergoes an extremely rapid development. This fact is demonstrated for the secretory immune system and HLA-DR in the intestinal mucosa and salivary glands (*63, 65, 76, 152*)

Predisposing factors (figur 9), e.g. genetic variants that imply unfavorable conditions may affect the infant in an unfavorable manner. A single gene or mutation causing SIDS has still not been found, but genetic risk factors that predispose to SIDS together with other factors most probably exists (*53*).

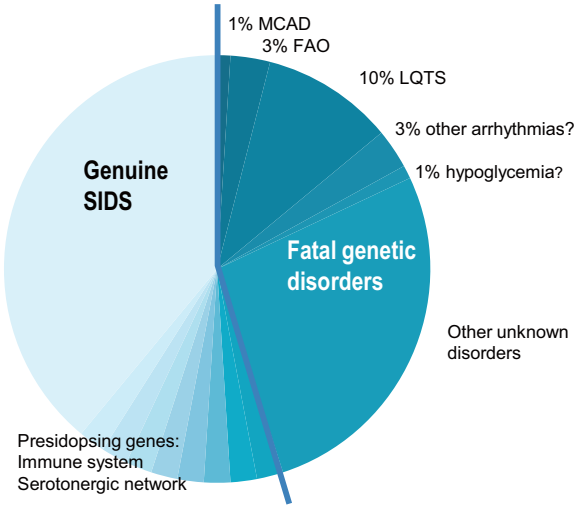


Figure 9: Possible causes of sudden unexpected infant death. A little less than half of the cases are most likely due to different conditions that cause death by themselves, such as MCAD deficiency and other fatty acid oxidation (FAO) defects (41-49), long QT-syndrome (55, 153-158), other arrhythmias, including defects in the genes encoding α-syntrophin (159), nitric oxide synthase 1 adaptor protein gene (NOS1AP) (160), caveolin-3 (CAV-3) (157, 161), glycerol-3-phosphate dehydrogenase 1-like gene (GPD1-L) (162) and ryanodine receptor RyR2 (163), and hypoglycemia caused by defects in the gene encoding the glucose-6-phosphate transporter (G6PT1) (164). The other cases are genuine SIDS, with genetic factors that may predispose to death in combination with external risk factors. These includes genes involved in the immune system, including IL-1, IL-6, IL-8, IL-10, TNFα, and complement component C4 (50, 52, 115, 117, 165-168), genes involved in brain development and function, including the genes encoding aquaporin 4 (AQP4), the human fifth Ewing variant (FEV), pituitary adenylate cyclase activation peptide (PACAP), paired like homeobox (PHOX) 2B, tyrosine hydroxylase (THO1) and the sodium/proton exchanger NHE3 (169-175), the serotonin transporter 5-HTT (176-179), and flavin monooxygenase 3 (FMO3), which is a gene involved in detoxification processes (180).

The trigger events that have got most attention since 1988 are prone sleeping, overheating and maternal smoking. After the "back to sleep" campaign in the late eighties and early nineties the SIDS rate have dropped dramatically. However, prone sleeping still remains a serious risk factor and more than 50% of the infants over 2 months are found prone sleeping (*181*). There are many hypothesis with regard to possible death mechanisms related to prone sleeping, e.g. suffocation due to positional asphyxia or rebreeding of carbon dioxide. The final proofs remain to be disclosed.

From epidemiological investigations as well as from studies on SIDS victims, it seems reasonable to assume that SIDS deaths occur as a result of three factors: a vulnerable developmental stage, predisposition, such as genetic risk factors and finally environmental factors that may act as triggers. When searching for patterns of genetic variants of significance, one is faced with a methodological challenges, one of these is the problem of multiple comparisons. This challenge is discussed in the following section.

6.7 Cytokine gene polymorphisms and its possible role in SIDS

The most likely candidates for explaining fatal vulnerability to ordinary harmless infections in SIDS, are the cytokine genes. The immune response is regulated by the cytokines and the presence and concentration of the different cytokines determine the duration and intensity of the immune response. The associations uncovered in this thesis between different polymorphisms and SIDS and environmental factors for SIDS, most likely represents only a small part of genetic patterns that may result in an unfortunate immunological reaction. The cytokine network is extremely complex and their study in deceased utmost complex. It is not possible to compare cytokine concentrations in dead subjects with levels measured in live patients. However, the stability of the cytokine concentration in CSF after death, is tested by performing several samplings in the post mortem period (*60*). These examination show that the concentrations partly increase after death, partly decrease. The median value was however fairly stable.

7 Conclusion

The present thesis indicates that there exists a predisposition for SIDS due to genetic variation in the cytokine network. Combined with environmental risk factors and a vulnerable developmental stage of the infant, this may be fatal. This means that a genetic predisposition by no means implies that SIDS will ensue. Most multifactorial diseases and conditions are not caused by just one unfortunate gene variation. Therefore, the development of multiplex chip analysis is becoming more and more important in research into genetic diseases. Although new knowledge has been obtained through the present studies, they also point to challenges of how to proceed in mapping genetic risk factors for SIDS. Last, but not least, we need to learn how to transform the knowledge into prevention of SIDS deaths. To sum up the conclusions for the thesis:

1. An unfavorable genetic profile in the TNFα gene may be involved in SIDS, by exposing the infant to both a high level of and a prolonged exposure to TNFα.
2. Genetic variation in the genes encoding IL-8 and IL-1β are a part of a genetic make-up that make infants sleeping prone at risk for SIDS.
3. Genetic variation in the IL-1 gene cluster may also contribute to SIDS, by giving a changed expression of both IL-1α and IL-1β and thus a disturbance in the immunological response.
4. SIDS cases with HLA-DR activation in laryngeal mucosa more often have rare genotypes of IL-1α, further strengthening the finding of an involvement of IL-1 in SIDS.
5. Taken together, the findings in this thesis indicate that specific interleukin gene variants may be predisposing factors for sudden unexpected infant death.

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9 Errata

1 Page 7 “that large a thymus” to “that a large thymus”

2 Page 10: text on the Y axis “ No of deaths pr 1000 live births”

3 Page 15: “SPA” to “SP-A”

4 Page 20: move “–“ to next line

5 Page 21: move “–“ to next line

6 Page 21: move “IL-“ to next line

7 page 28: “was created” to “will be created”

8 page 35: remove the red underscore from “IFNγ”

9 Page 35: change “IFNγ” to “IFN-γ”

9 Page 37: move “3’ ” to next line

10 Page 43: change “IL-1α VNTR A1A1/IL-1β+4845TT” to IL-1α VNTR A1A1/IL-1α+4845TT

11 There is an error in the dissertation sent to the medical faculty in my article ***“Is there any correlation between HLA-DR expression in laryngeal mucosa and interleukin gene variation in sudden infant death syndrome?”***were the illustration of a negative scored larynx was changed before submitted to the journal. This was regretfully not changed in the article that followed the dissertation. The illustration figure 1 a in the paper is changed to the same illustration as followed the submitted paper.

12 Double space at several location in the manuscript to single space.

13 No space after “period” to one space.

I

II

III

IV